

Potential Deleterious Effects of L-Citrulline Supplementation in Isoproterenol-Induced Myocardial Infarction: Focus on Nitrosative Stress

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ABSTRACT

L-Citrulline shows potential activity as a supplement to prevent myocardial infarction through vasodilative and possible antioxidative effects but may be deleterious by causing nitrosative stress. This study determined the potentially deleterious effects of L-citrulline supplementation in isoproterenol-induced myocardial infarction with a focus on nitrosative stress. L-Citrulline supplementation was given orally at dosages of 300 or 600mg/kg body weight daily for 6 days. Myocardial infarction was induced in Wistar rats via subcutaneous injection of isoproterenol (85 mg/kg body weight (BW)) on day 4 and 5. Blood pressure was measured at the end of the study (day 6) and rats were sacrificed to collect heart tissue samples for a histopathological evaluation. The histopathological evaluation was done using hematoxylin and eosin staining for the myocardial damage evaluation and immunohistochemical (IHC) staining of arginase-2, inducible nitric oxide synthase (iNOS), and 3-nitrotyrosine to evaluate nitrosative stress. L-Citrulline supplementation failed to show a significant protective effect on blood pressure and exacerbated the decrease of diastolic blood pressure. Both low and high dose L-citrulline supplementation had a significant protective effect on myocardial damage compared to the isoproterenol group ($p < 0.01$). L-Citrulline also caused increased nitrosative stress as shown by increased expression of arginase-2 and 3-nitrotyrosine on IHC staining but tended to show an ameliorative effect on iNOS expression. A significant increase in arginase-2 expression was detected between the high dose group and the other groups ($p < 0.01$ vs. normal and isoproterenol groups; $p < 0.05$ vs. low dose group). L-Citrulline supplementation increased 3-nitrotyrosine expression in a dose-dependent manner, which was significantly different compared to the normal group (low dose: $p < 0.013$; high dose: $p < 0.003$). L-Citrulline increased the production of nitrosative stress but resulted in less myocardial damage through its other effects.

Keywords: isoproterenol-induced myocardial infarct, L-citrulline, nitrosative stress

INTRODUCTION

Coronary heart disease (CHD) is a leading cause of death worldwide and acute myocardial

infarction (AMI) is the most lethal complication (Wong, 2014). AMI is caused by an imbalance between supply and demand of oxygen to the

myocardium (Burke and Virmani, 2007). Organic nitrates, such as nitroglycerin and isosorbide dinitrate, are medications used to treat this imbalance. These medications act by donating nitric oxide (NO), which causes vasodilation; thus, restoring the oxygen supply to the myocardium. Unfortunately, organic nitrates are not meant to be used constantly because they cause nitrate tolerance and provide a diminishing vasodilative effect (Münzel, *et al.*, 2013). L-Arginine is an amino acid in the endogenous NO cycle. L-Arginine supplementation increases the level of NO in the blood circulation by supplying a substrate used by endothelial nitric oxide synthase (eNOS), which is the principal mechanism of organic nitrates. L-Arginine has been demonstrated to reduce blood pressure in prehypertensive and hypertensive patients (Figueroa, *et al.*, 2017). L-Arginine causes no reduction of blood pressure in normotensive patients and can be used in a prolonged manner without causing nitrate tolerance. These effects alone show the potential of L-arginine as a supplement for patients at risk for CHD. L-Arginine also causes a reduction of monocyte adhesion and activity during inflammation (Adams, *et al.*, 1997). Combined with this effect, L-arginine is a potential supplement to protect the myocardium in the event of AMI. The drawback of L-arginine supplementation is that it has poor oral bioavailability. To overcome this problem, supplementation is given using L-citrulline, which is a precursor. Unlike L-arginine, L-citrulline does not undergo extensive first-pass metabolism and provides better oral bioavailability than L-arginine itself (Kaore, *et al.*, 2013).

Another potential drawback of L-arginine supplementation is that in the event of high oxidative stress, such as ischemia, it may cause an increase in the formation of peroxynitrite. Peroxynitrite is a nitrosative species that causes nitrosative stress (Muntean *et al.*, 2016). Coupled NOS uses L-arginine to form NO under physiological conditions. NOS is coupled with the help of tetrahydrobiopterin (BH₄), which is oxidized to dihydrobiopterin (BH₂) and loses its ability to couple with NOS. When uncoupled, NOS interacts with L-arginine to form peroxynitrite (Alkatis and Crabtree, 2012). During this phase, an increase in the level of L-arginine translates directly to an increase in peroxynitrite (Cardounel, *et al.*, 2005). Peroxynitrite is a mechanism used by monocytes during oxidative stress to produce inflammation. Monocytes produce inducible NOS (iNOS), which increases exponentially

during oxidative stress to help monocytes. Monocytes increase expression of arginase-II as part of inflammation-induced immunosuppression. Arginase-II converts L-arginine to L-ornithine to reduce the amount of substrate metabolized by uncoupled NOS (Munder, 2009). This study used isoproterenol as a means to induce AMI in rats. Isoproterenol (L-β-(3,4-dihydrofenil)-α-isopropylamino ethanol hydrochloride) is a medication used to treat heart failure based on its β-1 and β-2 receptor agonist activity. A high dose of isoproterenol increases the workload on the heart so that the oxygen demand of the myocardium outweighs the supply. The isoproterenol rat model has been extensively and reliably used to study the effect of substances on AMI (Liu *et al.*, 2013). This study investigated the potentially deleterious effects of L-citrulline supplementation on isoproterenol-induced myocardial infarction with a focus on nitrosative stress.

MATERIAL AND METHODS

This study used 24 male Wistar rats (weight 190-220g) obtained from the Health Research and Development breeding facility. This study was conducted according to the "Guide for the care and use of laboratory animals" and was approved by the Ethics Committee of The Faculty of Medicine, University of Indonesia.

This study was conducted in the animal laboratory of Universitas Indonesia, Faculty of Medicine, Department of Pharmacology and Therapeutics. The rats were housed at a room temperature of 25°C, humidity of 55%, and a 12h light/dark cycle. The rats were given standard pellets and water *ad libitum*. After a 1 week acclimation, the rats were randomized into 4 groups before the start of treatment: the normal group, which served as the sham group, receiving normal saline for MI induction and distilled water; the isoproterenol group, which served as the control group, receiving isoproterenol to induce MI and distilled water; and the low dose and high dose groups, receiving isoproterenol to induce MI and a daily dose of 300mg/kg BW and 600mg/kg BW L-citrulline (NutriVita, Lake Forest, CA, USA), respectively. The L-citrulline in this study was diluted in distilled water and given by gavage. MI was induced by subcutaneously injecting 85mg/kg BW isoproterenol on days 4 and 5 of treatment with a 24h interval between injections. The isoproterenol was reconstituted in normal saline before injection.

The rats were anesthetized with an intraperitoneal injection of a mixture of 80mg/kg BW ketamine and 8mg/kg BW xylazine. Anesthesia was given before blood pressure measurements were taken on day 3 of treatment and day 6 before the final measurement and euthanasia. Ketamine and xylazine were chosen due to their ease of use and minimal interference with the blood pressure measurement.

Blood pressure was measured under anesthesia on days 3 and 6 (end of treatment) of treatment using a CODA Scientific® non-invasive blood pressure device (Kent Scientific Corp., Torrington, CT, USA). Tissues were collected after the final blood pressure measurement was completed. Anesthetized rats were euthanized by supra cardiac puncture. After the rat was confirmed to be dead, a necropsy was done to collect the heart tissue sample. The dissected heart was washed with ice-cold saline, weighed, and photographed for documentation. A portion of heart tissue was cut and preserved in 10% neutral buffered formalin solution. The preserved heart tissue was then mounted in paraffin for hematoxylin and eosin (HE) and immunohistochemical (IHC) staining.

The HE stained heart samples were scored for myocarditis by a blinded pathologist. Scoring was done using a slight modification to the method used by Gibson-Corley, Olivier, & Meyerholz, (2013). Myocarditis was scored based on the percentage of myocarditis foci, which reflected severity. Based on the percentage, the score was divided into 5 categories: 0 non-existent, 1 mild (10%), 2 moderate (30%), 3 severe (50%), and 4 very severe (50–70%). The evaluation was done using a Leica DM 750 light microscope with 100x magnification (Leica Microsystems Inc., Buffalo Heights, IL, USA).

The histological sample was cut to a width of 3–5µm for IHC staining. After deparaffinization and rehydration, the sample was microwaved for 5min in 0.1M citrate buffer (pH 6.0). A solution of 3% hydrogen peroxide was added for 5min to remove endogen peroxides. The samples were then incubated with the respective antibodies: iNOS rabbit polyclonal antibody (1:200; Abcam Inc., Cambridge, MA, USA), arginase-II rabbit polyclonal antibody (1:100; Cell Signaling Technologies, San Diego, CA, USA), and 3-nitrotyrosine (1:500) in a phosphate buffered solution for 2h at room temperature in a humidity chamber and then overnight at 4°C. The FLEX Rabbit Negative Control, Ready-to-Use, (Dako Autostainer/Autostainer Plus; Dako, Carpinteria, CA, USA) was used as a

negative control. The secondary antibody was added to the sample and incubated for 1h at room temperature, followed by the addition of HRP-conjugated streptavidin and a 30min incubation. The proteins were visualized by adding 3,3'-diaminobenzidine (DAB) to the sample followed by a 10min incubation. The samples were stained with Harris hematoxylin, dehydrated, and mounted.

Regions of interest were evaluated. Protein expression was evaluated by comparing the percentage of area fraction stained with DAB as measured using ImageJ software (National Institutes of Health, Bethesda, MD, USA) with the immunohistochemistry toolbox plugin. The percentage of the area fraction was statistically analyzed (Jensen, 2013).

Parametric data were analyzed using one-way analysis of variance with Tukey's post-hoc test. Non-parametric data were analyzed using the Kruskal-Wallis test and the Mann-Whitney test. The data are expressed as mean \pm standard deviation for parametric data and as median (interquartile range) for non-parametric data. A p-value <0.05 was considered significant.

RESULT AND DISCUSSION

AMI caused by isoproterenol results in myocardial damage that impairs heart contractility. This impairment manifests itself in the form of lower systolic and diastolic blood pressure. To compensate for the hypotension, the blood vessels contract to maintain blood pressure. Systolic and diastolic blood pressure decreased post induction in all induced groups (Table I). All induced groups were significantly different from the normal group ($p < 0.05$). This result was in accordance with the study conducted by Anandan, *et al.*, (2015) where isoproterenol caused a decrease in blood pressure and protection against AMI was represented as an amelioration in this decrease. However, the decrease of blood pressure in both the low and high dose groups contradicted the supposed cardioprotective effect of L-citrulline against AMI. Because myocardial damage in both treatment groups was less severe than that in the isoproterenol group, these results were most likely caused by other factors besides failed protection. L-Citrulline inhibits this contraction by donating NO to promote relaxation of vascular smooth muscle. This condition followed the pharmacodynamic effect of L-citrulline supplementation in prehypertensive and hypertensive patients shown previously (Wong *et al.*, 2016).

Table I. Blood pressure measurements

Group	Systolic Blood Pressure (mmHg)	Diastolic Blood Pressure (mmHg)
Normal	177.44 ± 21.76	149.72 ± 21.35
Isoproterenol	106.94 ± 25.55*	91.83 ± 5.49*
Low Dose	108.08 ± 26.76*	87.73 ± 17.66*
High Dose	92.89 ± 9.18*	68.27 ± 3.14*

Data are presented as mean ± standard deviation; * p < 0.01 vs. normal group

Table II. Immunohistochemical Evaluation Summary

Group	Hematoxylin-Eosin Scoring	Arginase -2 (% of area)	iNOS (% of area)	3- Nitrotyrosine (% of area)
Normal	0 (0-0)	7.86±3.32**	1.33±1.24	3.68±2.38
Isoproterenol	4 (3.25-4)*	6.31±3.61**	3.24±2.57*	4.96±3.04
Low Dose	2 (1.25-2)*, #	8.45±3.98**	2.81±2.33*	5.66±3.03*
High Dose	2 (1.25-2)*, #	10.83±4.14	2.25±1.96	6.67±4.06*

Data are presented as median (interquartile range) and mean ± standard deviation; *: significant difference vs. normal group (p < 0.05); # (p < 0.05) vs. isoproterenol group; **Significant difference vs. high dose group (p < 0.05)

These effects might explain why L-citrulline supplementation did not prevent the decrease of blood pressure and tended to exacerbate it instead. However, these same effects may not necessarily help in the aftermath of AMI. Inhibited vasoconstriction, combined with decreased heart contractility may cause hypotension and hypoperfusion of organs, which can lead to organ failure and death (Metra *et al.*, 2009).

Isoproterenol caused myocardial damage, which was evaluated by scoring the percentage of myocarditis in the histopathological samples. Isoproterenol resulted in higher scores (Table II). The groups receiving isoproterenol scored significantly higher than the normal group (p < 0.01). A significant difference also occurred between the groups receiving low and high dose L-citrulline compared to the isoproterenol group (p < 0.01; Figure 1). Isoproterenol caused significant damage across all induced groups, while the normal group remained free of damage, indicating that induction of AMI by isoproterenol was successful, as shown previously (Filho *et al.*, 2011). L-Citrulline supplementation failed to completely prevent the damage caused by AMI but ameliorated some damage as shown by the significantly lower myocarditis score compared to the isoproterenol group.

L-Citrulline supplementation increased arginase-2 in a dose-dependent manner compared to the groups that were not supplemented. A significant increase was found between the high

dose group and the remaining groups (p < 0.01 vs. normal and isoproterenol groups; p < 0.05 vs. low dose group) (Table II). Induction of AMI with isoproterenol without L-citrulline supplementation resulted in slightly lower arginase-2 expression compared to the normal group although the difference was not significant. A histological representation of the samples (Figure 2). A decrease of arginase-2 expression during inflammation is necessary to help monocytes produce inflammatory cytokines, namely peroxynitrite, using uncoupled iNOS (Schlüter, *et al.*, 2015). This might explain why the expression of arginase-2 was slightly lower in the isoproterenol group than that in the normal group. In contrast, increased arginase-2 expression during inflammation inhibits the production of nitrosative stress. This increased expression might be excess arginase-2 that was present before induction as a physiological response to metabolize excess L-arginine in circulation. A more probable explanation is that monocytes expressed arginase-2 to control inflammation at a rate comparable to the level of circulating L-arginine to prevent overproduction of peroxynitrite. This might explain the increase of arginase-2 in groups receiving L-citrulline supplementation.

Isoproterenol administration resulted in significantly higher expression of iNOS in the isoproterenol and low dose groups compared to the normal group (p < 0.01; Table II).

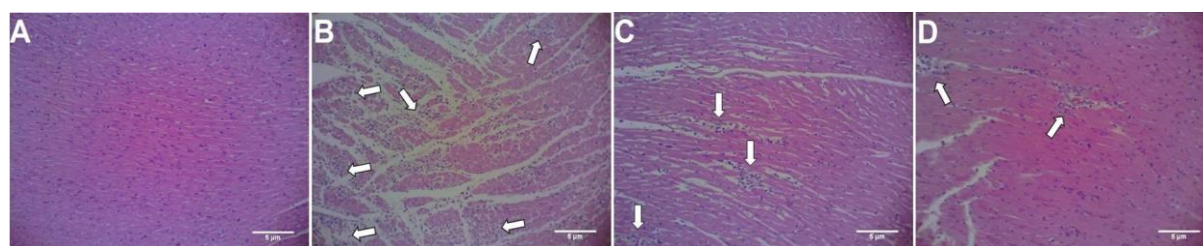


Figure 1. Heart tissue (100x magnification; hematoxylin and eosin stain). A. Normal group; B. isoproterenol only group; C. isoproterenol + low dose L-citrulline group; D. isoproterenol + high dose L-citrulline group. White arrows are myocarditis foci, reflecting the severity of the myocardial infarction induced by isoproterenol. L-citrulline supplementation (C, D) can be seen ameliorating the number of myocarditis foci.

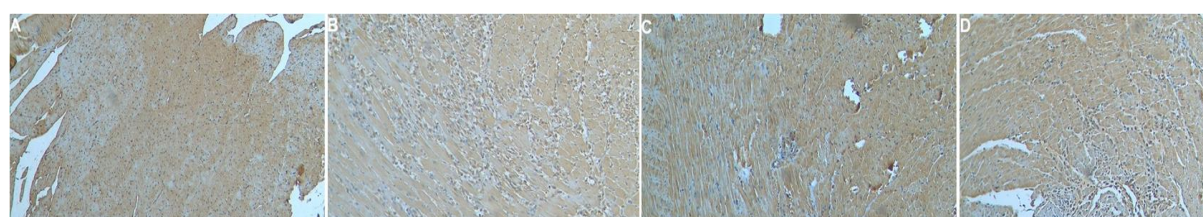


Figure 2. Heart tissue (100× magnification; arginase-2 staining). A. Normal group; B. isoproterenol only group; C. isoproterenol + low dose L-citrulline group; D. isoproterenol + high dose L-citrulline group.

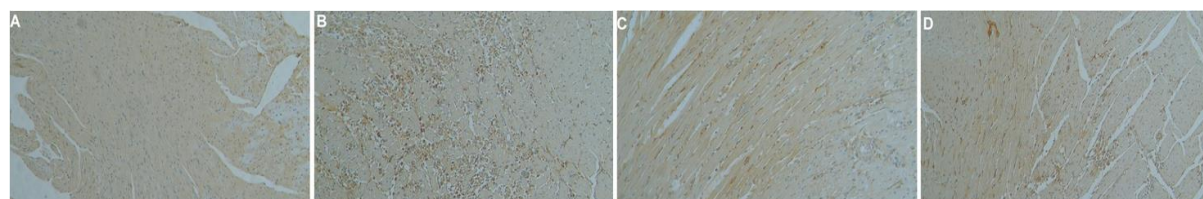


Figure 3. Heart tissue (100× magnification; inducible nitric oxide synthase staining). A. Normal group; B. isoproterenol only group; C. isoproterenol + low dose L-citrulline group; D. isoproterenol + high dose L-citrulline group.

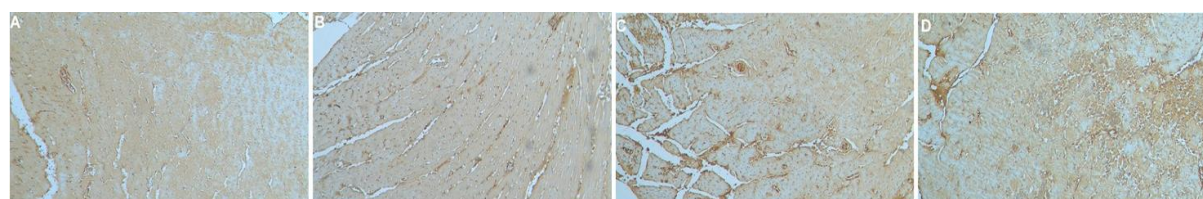


Figure 4. Heart tissue (100× magnification; 3-nitrotyrosine staining). A. Normal group; B. isoproterenol only group; C. isoproterenol + low dose L-citrulline group; D. isoproterenol + high dose L-citrulline group.

High dose L-citrulline tended to ameliorate the increase of iNOS expression compared to that in the isoproterenol and low dose groups. Histological representation of the samples can be seen in Figure 3. iNOS expression was elevated in all induced groups. L-Citrulline supplementation tended to

ameliorate the increased expression in a dose-dependent manner. The low dose group revealed a lower percentage of surface area compared to the isoproterenol group but it was significantly different from the normal group. iNOS expression in the high dose group tended to be higher than that

in the normal group but the difference was not significant. iNOS expression increases during AMI as part of the inflammatory mechanism (Liu et al., 2013). The lower level of iNOS in the groups that received L-citrulline supplementation could have been caused by the reduced number of monocytes expressing iNOS, as a pharmacodynamic effect of an L-arginine surplus in circulation inhibits monocyte adhesion (Adams *et al.*, 1997).

3-Nitrotyrosine was used as a marker to measure nitrosative stress. Isoproterenol administration significantly increased the expression of 3-nitrotyrosine in all induced groups compared to the normal group (Table 2). L-Citrulline supplementation caused a significant increase in 3-nitrotyrosine expression in a dose-dependent manner compared to the normal group (low dose: $p < 0.013$; high dose: $p < 0.003$). A histological representation of the sample is shown in Figure 4. The increase in 3-nitrotyrosine expression was caused by nitrosative stress that occurred with oxidative stress during ischemia/reperfusion that caused AMI. The level of 3-nitrotyrosine in groups receiving L-citrulline was even higher than that in the isoproterenol group in a dose-dependent manner. This increase of 3-nitrotyrosine was caused by the increased amount of peroxynitrite produced because of the increased level of L-arginine in circulation. This result indicates that nitrosative stress during ischemia/reperfusion is exacerbated by L-citrulline supplementation, as reported previously (Mori, *et al.*, 1998).

Damage that resulted in AMI occurred because of the oxidative and nitrosative stress generated during ischemia/reperfusion. Both the low dose and high dose groups exhibited less myocardial damage but higher nitrosative stress than that in the isoproterenol group. This may have been caused by a shift in stress causing the damage. Increased nitrosative stress is caused by a shift of superoxide from forming H_2O_2 that caused oxidative stress and reacted with free NO to form peroxynitrite. This shift resulted in less total myocardial damage.

CONCLUSION

We conclude that L-citrulline supplementation in isoproterenol-induced MI caused more nitrosative stress but less myocardial damage. The lower myocardial damage may be partially attributed to a reduction in iNOS-producing monocytes and the vasodilative effects of L-citrulline supplementation. However, the

persistent vasodilative effect combined with decreased cardiac output may cause hypotension and hypoperfusion of organs. This study provides information regarding the potential benefit and risk of using L-citrulline supplementation to prevent AMI.

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